**Quick stitch: vignetting correction of large immunofluorescence-based mosaics with high-pass filtering and recursive normalization**

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Availability: Compiled executable for PC and mac, as well as source available at the Zartman lab website at: <http://www3.nd.edu/~jzartman/resources/index.html>

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**Abstract**

Fluorescence micrographs naturally exhibit darkening around the edges (vignetting), which makes seamless stitching challenging. When vignetting is not corrected, the stitched image will have visible seams where the individual images (tiles) overlap, introducing a systematic error that makes segmentation and morphometric analysis challenging. While multiple methods exist to correct for vignetting, there currently is a lack of an open-access tool for researchers that robustly handles large 2D immunofluorescence-based mosaic images. Here, we develop and validate the QuickStitch tool, which applies a recursive normalization stitching algorithm to stitch large-tissue immunofluorescence-based mosaics without incurring vignetting seams. We demonstrate how the image processing pipeline is shown to work with tissues of differing size, morphology, and intensity, and requires no specific information about the imaging system. The vignetting correction tool is provided as open-source tool that is both user friendly and extensible to incorporation into existing image processing pipelines. This enables studies that require accurate segmentation and analysis of high-resolution datasets when parameters of interest include both cellular-level phenomenon and larger tissue-level regions of interest.

**INTRODUCTION**

In immunofluorescence microscopy studies of large-scale tissue morphology, mosaics comprised of multiple fields of view are often required to obtain high-resolution data necessary to study cell-level properties. For example, quantitatively connecting cell shape parameters to tissue-level morphogenetic processes is a key challenge in developmental biology. Due to the uneven illumination of optical systems present in all optical equipment (1), objects located near the edges of fluorescence micrographs have a lower signal intensity than those in the center of the field of view. This causes stitched montages to have visible seams (vignetting) that reduce the accuracy of image data (2). While systems exist to reduce vignetting by providing more uniform illumination, including Borealis - Perfect Illumination Delivery™, vignetting is never entirely eliminated. A solution is needed to generate seamless mosaics of fluorescent images of large tissues with sparse background, such as histological samples.

Further, there currently is not an off-the-shelf tool to our knowledge that is available for automated, parameter-free correction of vignetting without using background information. To develop a practical solution for large scale tissue-based data sets at the cellular scale, we have developed an open-source, freely available, computationally efficient image processing tool that applies bandpass Fast Fourier Transform (FFT) to individual tiles then utilizes optimal normalization to enable seamless stitching. The method presented here achieves seamless stitching in two steps: first, vignetting artifacts present in individual tiles are removed by FFT high-pass filtering; second, a recursive normalization algorithm is used to achieve even image intensities in adjacent tiles. The FFT is a computational algorithm commonly used to decompose sequences into a sum of sinusoidal functions (16). Thus, FFT high-pass filtering can be used to remove low frequency objects affecting the entire field of view, such as vignetting artifacts, while preserving the high frequency features that comprise the raw image.

Here we validate the Quick stitch tool on whole-mount *Drosophila* embryos.

Several flat-field correction methods are commonly used to approximate the original pattern of a signal from the raw data collected. The flat-field correction equation for vignetting is given by:

|  |  |
| --- | --- |
|  | (1) |

where and are the original and corrected image matrices respectively, is the vignetting mask, which is matrix the same size as the image matrix, and is its spatial average of the vignetting mask, and is the background intensity (3). The indices and represent the position of pixels in the and directions. The background intensity is frequently assumed to be constant across the field and hence, does not contribute to vignetting artifacts.

**The need for an open-source vignetting tool for multiplexed, large-scale mosaics.** Existing flat-field correction methods generally utilize Equation 1 and rely on obtaining and through the use of a variety of methods that are summarized below:

**Fluorescent correction slide:** A common recourse for flat-field correction is to obtain an image of a blank area on a slide to utilize as a vignetting mask (3–6). This image is assumed to be the inverse of , while is set to zero in Eq. 1. This approach is applicable for brightfield microscopy; however, in the case of fluorescence microscopy, a fluorescent slide must be used because the empty region of the glass slide does not yield a fluorescence signal (3). Furthermore, the assumption of to be zero causes artifacts in the interpretation of fluorescence data, which results in the over or under-estimation of regional concentrations (7). To avoid this is assumed to be an image taken while the lasers are inactive. The correction slide method for flat-field correction also makes the assumption that the vignetting mask is the same for all images, regardless of the signal intensity (3–5). Is there an open source-implementation? Why is not suitable for our data?

**Background segmentation:** Background segmentation techniques can be used to correct for vignetting after image acquisition via the creation of a vignetting mask from background information, typically by averaging or taking the median of background pixels (8). However, this information may not be present if the sample takes up the whole viewing area, or may be difficult to segment in whole-tissue mosaics given that different tissue-types and cell cultures frequently require tailored segmentation methods. Such background detection methods assume that the vignetting mask is dependent only on position and not intensity, enabling the user to obtain a background image from a composite of multiple fields of view (8,9). As a result, this requires segmentation to separate the background from the foreground, which is difficult to do for features of varying size, because an entirely new segmentation algorithm is needed for each unique application. Is there an open source-implementation? Why is not suitable for our data? MicroMos is an open-source background segmentation tool that was principally designed for brightfield microscopy. We tested our datasets using MicroMos, we encountered errors in registration and did not accurately segment the background and foreground.

**Physical (parametric) principles:** Another class of methods for vignetting correction is to solve for the vignetting mask using analytical solutions derived from the physics governing vignetting. Generally, focal length, principal point, aspect ratio, and skew of the lens must either be provided or measured from reference material. Good approximations of the vignetting function can even be made without a reference slide if the geometry of the optical instrumentation is known such that these parameters can be obtained. Since precise specifications which govern every piece of optical equipment involved in the imaging are needed, this method is most feasible for applications by the companies that supply the optical equipment. Highly informed parametric methods are also often slower than other methods. Further, it is impossible for a parametric method to account for all sources of vignetting. (10,11). Is there an open source-implementation? Why is not suitable for our data?

**Image averaging methods:** For image averaging methods, a set of images is averaged to obtain an “average image.” The inverse of the average is taken to be the vignetting mask. This method does not require a reference slide; however, it assumes that objects in the image are uniformly distributed, meaning that a large number of reference images are needed if the landmarks are not sufficiently homogeneous (12). Is there an open source-implementation? Why is not suitable for our data?

**High-pass filtering methods:** Frequency-filtering methods decompose an image into a sum of images of various spatial frequencies, such that components of the signal

* What is a high-pass filter, and why is vignetting a low-frequency phenomenon
* How FFT works
* How Gaussian works (14,15)
* Why these methods alone are not suitable for mosaicking and there is a need for normalization prior to mosaicing

Although FFT is more frequently employed in image denoising algorithms (13), here we utilize FFT filtering of the tiles to remove features lower in frequency than the cutoff frequency, which is greater than the frequency of vignetting across an empty field, but less than the frequency of the largest object. Because only high-frequency images persist in the final summation, this is a high pass FFT filter. Consequentially, a basic assumption of this approach is that vignetting effect has a larger period than other features in the images. This results in the removal of vignetting artifacts because vignetting is a low-frequency phenomenon.

**Need for normalization method**

In the course of obtaining high resolution, multi-tiled immunofluorescent image data sets of late stage *Drosophila* embryos, we found that visible seams caused by vignetting were present. We tested several vignetting correction methods previously described in the literature in an effort to correct for vignetting to produce clearly defined mosaics with image features of variable characteristic sizes. We concluded that none of the approaches satisfactorily correct for vignetting. In particular, we compared our proposed method to the rhodamine fluorescence slide method, and found that our method was best able to qualitatively eliminate the seams arising from vignetting (Future Figure ?).

**MATERIAL AND METHODS**

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**Immunohistochemistry, confocal imaging and rhodamine test slide generation**

A *Drosophila* line expressing GAL4 under the engrailed (*en*) promotor and CD8::GFP under the UAS promotor was used in preparing Fig. 2. The immunohistochemistry (IHC) protocol was based on previously described experiments optimized for dpERK labeling (23,24) with rabbit anti-dpERK (1:100, Cell Signaling), rat DCAD2 (1:100, DSHB) and DAPI (5 µg/ml, Invitrogen DU1306), with goat anti-rat IgG 561 (1:500, Invitrogen), and goat anti-rabbit IgG 647 (1:500, Invitrogen). An Andor spinning disc confocal microscope with a piezo stage at 1.0 µm intervals was used to collect confocal z-stacks. Six by six grids with thirty-three percent overlap were collected for each of the four channels for each embryo. The same settings were used to obtain images of the Rhodamine test slide. Blank images were generated without fluorescent media. MetaMorph® version 7.0.11 was employed for image collection. A saturated Rhodamine (supplier) solution in phosphate-buffered saline was filtered with a syringe filter and placed on a glass slide and sealed with fingernail polish.

Here, a recursive algorithm linearly normalizes adjacent transformed tiles to minimize the intensity difference of overlap pixel intensities (Fig. 2A). The recursive nature of the algorithm results in linear scaling with time (Fig. 2B).

**FFT filtering**

Prior to normalization, images were processed to remove vignetting artifacts. A maximum-intensity z-projection of each z-stack was generated using Miji (17), a MATLAB implementation of Fiji is just ImageJ (FIJI) (18). For the dpERK antibody stainings, a median filter of radius 3 pixels was employed to remove salt-and-pepper noise caused by the low intensity of the signal relative to the background. The FFT high-pass filter in FIJI was utilized to eliminate vignetting artifacts present in individual tiles. A qualitative parameter sweep revealed that a large object filter between 80 and 150 pixels was sufficient to remove the vignetting artifacts while preserving small image features.

**Gaussian Filtering**

As an alternative to FFT, images were processed with a high-pass Gaussian filter:

|  |  |
| --- | --- |
|  | (2) |

where is the processed image, is the raw image, and is the sigma radius of the Gaussian filter.

**Normalization**

In order to compare or stitch multiple transformed images, objects of similar intensity must be registered as having the same signal strength. We implement a recursive algorithm to simultaneously normalized adjacent sets of an increasing number of tiles. In each recursion step, we linearly rescale sets of adjacent tiles:

|  |  |
| --- | --- |
|  | (2) |

Here, and are independent of and , and and represent grid coordinates of tiles within a mosaic. The parameters and are chosen such that the intensity differences to neighboring tiles are minimized in the overlapping region. At each recursion step we consider up to 2x2 sets of adjacent tiles (Fig. 1A). We minimize a cost function using global search algorithm employing the *fmincon* local solver in MATLAB R2015a in order to minimize the intensity differences in the overlap (19). The cost function takes different forms depending on whether the overlap contains two (2x1 or 1x2) or four (2x2) tiles. For the case of the two adjacent sets of tiles the cost function is

|  |  |
| --- | --- |
|  | (3) |

where and denote the intensity of pixels at the edges of the two overlapping tiles, while and are the optimized parameters for their respective tiles. The resulting parameters are used to modify the tiles using Eq. 2. Importantly, the optimization underlies the constraints that must be greater than one, and must be greater than zero in order to avoid negative intensities, and reduce losses in precision. The cost function for the remaining cases is constructed similarly. To reduce the computational cost of the optimization, intensities in Eq. 3 are binned to one-fourth resolution.

**Algorithm testing**

We validated the image processing pipeline on 24 late stage Drosophila embryos expressing CD8::GFP under the *engrailed* enhancer. Engrailed is expressed in the posterior compartments of embryonic segments (20). This marker was chosen to test the method because bands of Engrailed expression are frequently larger than one field of view, allowing the effect of RNS on large features to be studied. IHC was used to visualize cell boundaries (using DE-cadherin) in order to obtain cell-level morphological information and assess the ability of RNS to correct vignetting in cases of small details. Further, measurements of gene regulatory molecules are important for understanding the mechanisms behind cell-signaling and other developmental processes. Doubly phospho-ERK (dpERK), the downstream target of the Epidermal Growth Factor Receptor (EGFR) pathway, is important for many developmental functions including embryonic compartment size homeostasis (21,22). dpERK was assayed, corrected and stitched in order to assess the ability of RNS to correct vignetting of non-homogeneous multi-cellular features that typically is at a lower intensity.

**RESULTS AND DISCUSSION**

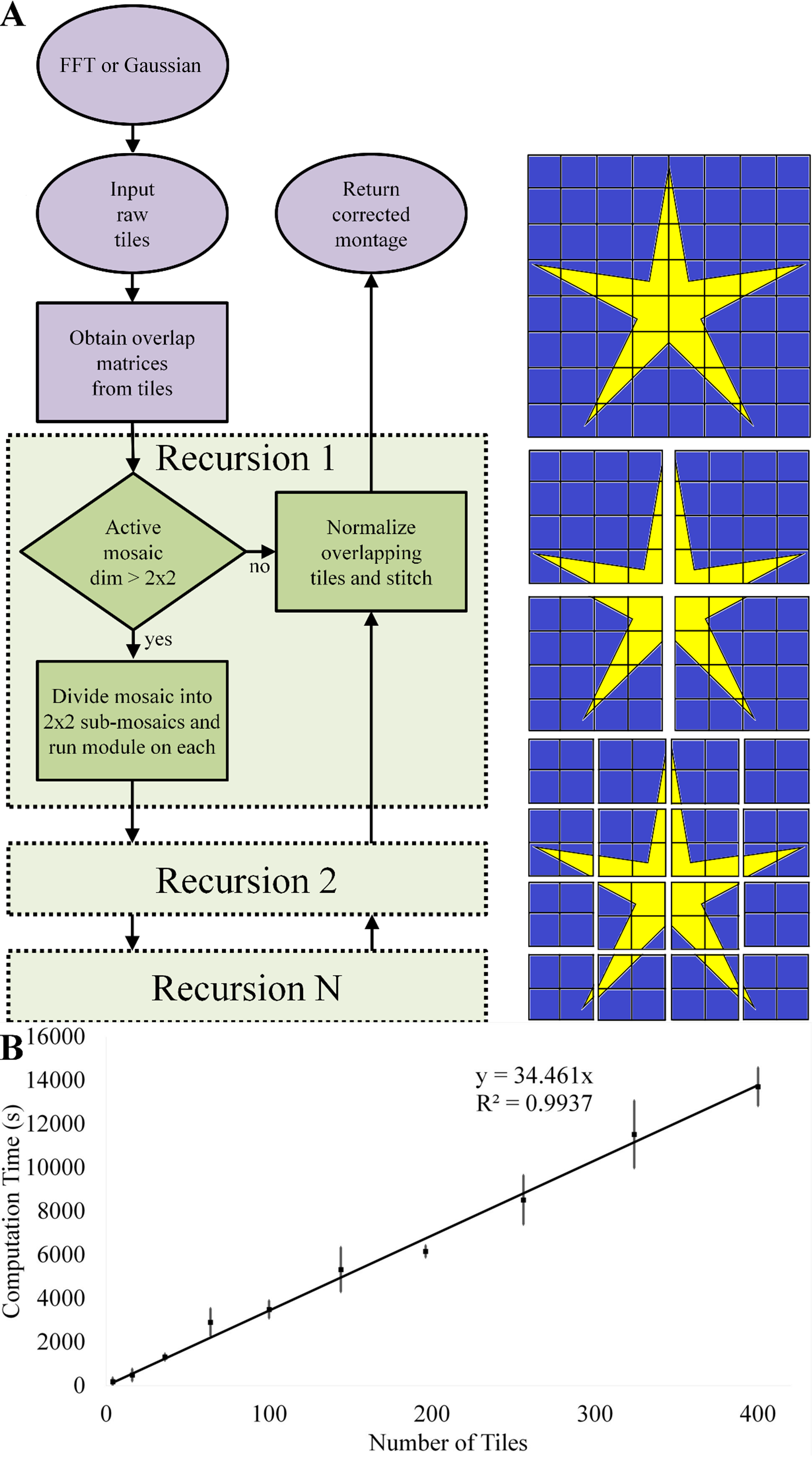
FFT is an algorithm that is used by this application to decompose raw images into a summation of component images characterized by differing frequencies. Normalization is needed because use of FFT filtering determines the shape of the vignetting artifact, but not the absolute intensity.

RNS successfully removed vignetting artifacts from *Drosophila* embryo mosaics (Fig. 2). By removing vignetting artifacts from cell-boundary images (Fig. 2C′), watershed segmentation applications such as Seedwater Segmenter (25) and custom segmentation software could quickly be used to segment cell boundaries for the whole embryo with fewer errors in automatic seed selection, as cellular minima occur in a narrower spatial range, and with fewer errors in boundary identification, as cell boundaries will exist in a narrower intensity range. The *en*>GFP signal (Fig. 2B), which represents lineage-restricted compartments in the embryo, is an example of a large-scale pattern or structure within the tissue useful for cell classification. Cell-fate classification by thresholding *en* is dependent on a uniform signal, and will result in fewer classification errors with this correction (Fig. 2B′). Even features with low intensities such as the spatial pattern of dpERK antibody staining (Fig. 2A) were preserved through the normalization process. By correcting for vignetting, the pattern of dpERK activity across multiple tiles is much more apparent to the eye, and matches previous low resolution reports of dpERK expression (21,23). For example, after correction, the characteristic tracheal pit pattern is visible (Fig. 2E, 2E′, Gabay, Seger, & Shilo, 1997). This demonstrates the value of vignetting correction for observing phenomenon where the magnitude of the signal is comparable to the magnitude of vignetting artifacts such as high-magnification imaging of the low-intensity gradient of dpERK.

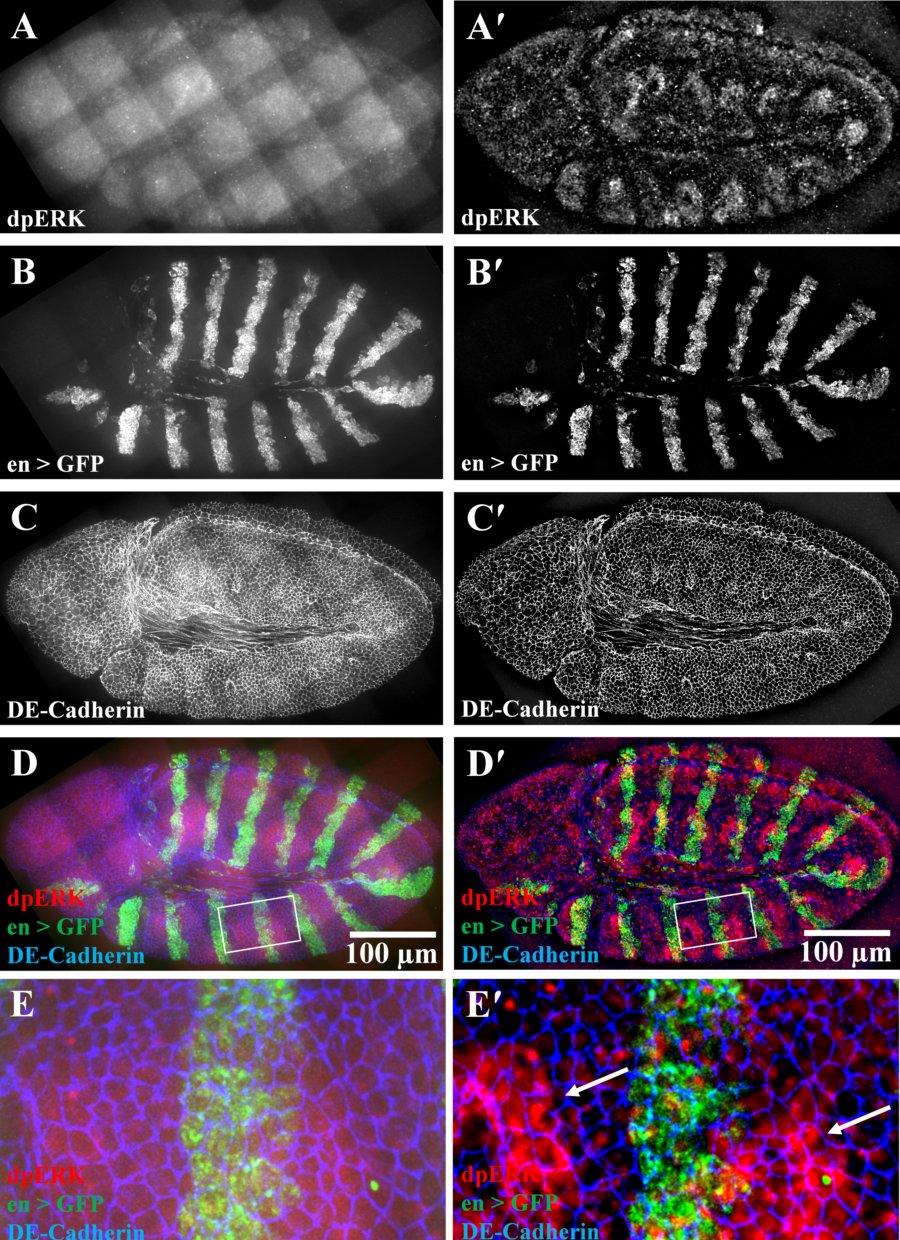
RNS is an efficient and preferable over background segmentation algorithms in cases where the tissue makes up the majority of the field of view where little background information is available such as histological imaging or confluent cell cultures. RNS relies on the assumption that vignetting is a low-frequency phenomenon, meaning that it can be removed by using a FFT high-pass filter. A basic assumption of this method is that features are smaller than the cutoff frequency. This implementation was developed for two-dimensional z-projections of confocal stacks where z-slices do not have an overlap in the z direction. RNS is available online as a pre-compiled executable for Macintosh OS X Yosemite and Windows 8.1 operating systems and does not require a MATLAB license to use.

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**Fig. 2: Overview of image processing method.** A) The flow diagram shows how a multi-tile mosaic is processed after vignetting correction to normalize tiles and enable subsequent stitching into a mosaic. Mosaics of size 2x2 or smaller are optimized to minimize the difference in the overlaps, as defined by Eq. 3. Mosaics larger than 2x2 are subdivided into sub-mosaics in a 2x2 configuration, and each sub-mosaic is processed in a recursive instance of the script. The normalized sub-mosaics are then normalized through the 2x2 optimization using Eq. 3. As a result, no optimization is ever run with more than six independent parameters, and computation time of mosaics of any size scales nearly linearly with the number of overlaps. B)The correction was run on montages with dimensions MxM, with M ranging from 2 to 20 in multiples of two, where 6 (or a total of 36 tiles) represents the dataset tested in this study. The image used contained a linear gradient, divided into tiles with 10% overlap. The tiles were multiplied by a fourth order cosine function to simulate vignetting, and Gaussian noise with a standard deviation of 10% was applied. The resulting data showed a linear correlation between the number of tiles stitched and computation time. N = 5 for each condition, error bars represent standard deviation.



**Fig. 3. Vignetting correction of a multiplexed immunofluorescent-stained *Drosophila* embryo.** (A-E) Uncorrected and stitched confocal z-projections of a stage-11 *Drosophila* embryo. All three channels show vignetting. (A) For dpERK, which has a low signal, the effect is very strong. (B) Engrailed is localized in the posterior compartment of segments. (C) DE-Cadherin marks the boundaries of cells. (A′-E′) The same images, corrected and normalized using our RNS algorithm. Images have a resolution of 2436x1284 pixels, allowing for high precision cell-level analysis. (E′) Arrows represent the characteristic tracheal pit pattern where dpERK is highly concentrated at this stage of development that is revealed by RNS.

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